IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Patent Application of **WILLIAMS et al.**	Atty.Ref: **CTH-03 (5439)**
Serial No. 09/600,060	Group Art Unit: 1644
Filed: 07/10/2000	Examiner: **P.N. Huynh**

For: AGENT FOR TREATING ALLERGIC OR HYPERSENSITIVITY CONDITIONS

DECLARATION UNDER 37 C.F.R. Sec. No. 1.132

I, Dr. Neil Andrew Williams, hereby declare and state as follows:

I am a British Subject and co-inventor of the above mentioned US patent application having a priority date of 9 January 1998. I am a faculty member at the University of Bristol, UK. My professional qualifications are summarized at the end of this document following my signature. I submit this declaration in connection with issues raised by the Examiner in relation to an Office Action dated 7/16/2002 in relation to the above-referenced US patent application.

The present invention concerns applications of technical aspects of *E. coli* enterotoxin.

Contrary to expectations based on the published art, an agent that is capable of binding to GM1 and mediating GM1-associated signalling events, is able to inhibit an allergic response in a well recognised animal model of asthma and allergic rhinitis. For our studies we have utilised the B-subunit of Escherichia coli heat-labile enterotoxin (EtxB), which is an agent known, like its relative cholera toxin B-subunit and the whole toxins, Etx and Ctx, to bind with high affinity to GM1. We demonstrate that administration of EtxB to mice that have previously been sensitised to produce IgE antibodies to ovalbumin, is able to dramatically reduce the levels of cellular infiltration into the lung following a subsequent aerosolised challenge with ovalbumin. Cellular infiltration into the lung, especially of eosinophils, is a recognised marker of the severity of the allergic response in this

model and is a feature of human allergic airway disease. Importantly, the changes in cellular infiltration which follow the administration of EtxB are associated with a decrease in the levels of IgE antibodies against ovalbumin and a reduction in the levels of interleukin-4 in the lungs and in lymph node cell responses to ovalbumin. It is noteworthy that IgE antibodies are the primary mediators of the allergic response in asthma and allergic rhinitis and that the production of IgE by the immune system is specifically regulated by interleukin-4. Further, these same immunological processes are causative in the pathology of a wide variety of other allergic diseases in man, suggesting that EtxB may be similarly active in other conditions. The data also show that similar reductions in the allergic response can be achieved when treatment combines the administration of EtxB mixed with, but not conjugated to, ovalbumin (the allergen in this system).

The Following Experiments describe the usefulness of EtxB in the Treatment of Allergic or Hypersensitivity Conditions.

EtxB allergy experiment

Sensitization

Mice were sensitized to ovalbumin (OVA) (10 μg/injection) adsorbed to 1.5 mg Al $(OH)_3$ by intra-peritoneal injections on day 1, 14 and 21. Control mice were immunized with PBS alone.

Challenge

Animals were challenged on days 27 and 28 by ultrasonic nebulization of 1% OVA (chicken OVA, grade V, Sigma Chemical Co., St. Louis, Mo.) diluted in sterile PBS. For this purpose, five mice were placed into a clear plastic box with a re- movable top (dimension 22 by 23 by 14 cm). The OVA solution was aerosolized into one end of the box with an ultrasonic nebulizer and a continuous pressure of 5psi. At the other end of the chamber were two small air holes to ensure a continuous cross-current of air flow.

Experimental groups (n=10/group)

Group 1: Sensitization negative control (PBS i.p.)

Group 2: Sensitization positive control (i.p. OVA; untreated)

Group 3: EtxB alone (i.p. OVA; i.n. EtxB 20μg i.n. days 14, 16, 18, 20)

Group 4: EtxB + OVA (i.p. OVA; i.n. EtxB 20μg + OVA 20μg i.n. days 14, 16, 18, 20)

Endpoints

Animals were sacrificed 24 hours after the last airway allergen challenge and sampling was a follows.

1. BAL: The trachea was exposed and cannulated. BAL was performed by two lavages with 0.8ml ice cold PBS, which were pooled and the volume and total cell number were determined. Cytospins were prepared for each sample by centrifugation of 50μl BAL fluid. These were fixed in acetone and stained with Diff Quick. Differential counts of 2 x 100 cells were performed classifying the cells as either neutrophils, eosinophils, lymphocytes or macrophages. Cell free lavage fluids were stored for later analysis. Thawed sterile bronchoalveolar lavage BAL fluid was tested for the presence of cytokines (IL-4, IL-10, γIFN) by ELISA. Quantities of the cytokines were calculated using linear regression analysis.

2. Nasal wash: Reversal of the tracheal canula allowed 0.5ml PBS to be drawn through the nose to wash the nasal mucosa for antibodies. The volume was noted and the samples frozen for later antibody analysis.

3. Blood: Animals were bled by cardiac puncture under terminal anaesthesia. The blood was allowed to form a clot and the serum was removed following centrifugation. Serum samples were frozen for analysis later.

4. Antibodies: Nasal washes were analysed for the presence of anti-OVA IgA levels. Serum samples were analysed for the presence of anti-OVA total Ig, IgE, IgG1 and IgG2a levels. Antibody analysis was carried out by standard ELISA. Bourel

fluids were analysed for the presence of anti-OVA IgE. Data was analysed by linear regression analysis.

Results

Figure 1. The effects of EtxB on total cell infiltration into the lung.

The data represent counts of total cells/BAL fluid recovered for each group. The data clearly show that injection of OVA in alum sensitizes Balb/c mice to lung cell infiltration following exposure to aerosolized OVA. Treatment with either EtxB alone or EtxB + OVA dramatically suppresses the allergic response in the BAL.

Figure 2. The effects of EtxB on total cell infiltration into the lung II.

The data show the results of analysing the numbers of individual leukocyte populations infiltrating the lungs after exposure to aerosolized OVA. Differential counts of cytospins prepared from BAL samples were used for the analysis. The results clearly indicate that EtxB treatment suppresses infiltration of each cell type into the lung. Importantly, the most dramatic difference is in the levels of eosinophils present in the BAL fluid. Eosinophils are the major cell population which respond to the presence of IgE in the lung and release inflammatory mediators.

Figure 3. Effects of EtxB treatment on the levels of OVA-specific IgE in BAL fluids.

Samples of BAL fluid from individual mice were analysed using doubling dilutions for the presence of anti-OVA IgE by specific ELISA. Linear regression analysis was used to determine endpoint titres, which are shown for each animal (squares) along with the median for the groups (bar). The data demonstrate that EtxB and EtxB + OVA can modulate local IgE levels in the lung.

Figure 4. Effects of EtxB treatment on the levels of cytokines in BAL fluids

The presence of IL-4, IL-10 and γIFN in individual BAL fluids from mice was determined by ELISA. The quantity of each cytokine present was calculated by linear regression analysis as compared to a standard curve obtained with recombinant cytokine. The data indicate that sensitzation with OVA in alum leads to an elevation in the quantities of IL-4 (the major cytokine associated with the generation of IgE). IL-10 and γIFN was detected in the negative controls as well as in the positive controls. Treatment with EtxB or EtxB + OVA dramtically suppressed the levels of IL-4 in BAL. EtxB may have raised the levels of IL-10 (a key regulatory cytokine) however the wide variation in the levels detected in individual mice precludes a strong conclusion being drawn on this point. Squares represent levels of cytokine in individual mice and the bar is the median for each group.

Figure 5. Effects of EtxB treatment on the levels of OVA-specific IgA in nasal washes

It is conceivable that EtxB may have prevented clinical asthma by switching the nature of the anti-OVA immune response away from a pro-IgE Th2 response and toward a mucosal IgA response. If this were the case, then it would be predicted that anti-OVA IgA levels would be raised in the nasal secretions. Analsysis of nasal anti-OVA IgA by specific ELISA revealed that EtxB treatment did not alter the levels of mucosal antibodies. In contrast, treatment with EtxB + OVA produced a substantial rise in anti-OVA IgA levels. This indicates that while both EtxB alone and EtxB + OVA were effective at reducing cell infiltration into the lung as well as altering local IgE and cytokine production, the mechanisms of immune modulation in the presence of the priming antigen are markedly different. Squares represent levels of cytokine in individual mice and the bar is the median for each group.

Figure 6. Effects of EtxB treatment on the levels of OVA-specific IgE in serum

Levels of anti-OVA IgE were measured in samples of serum by specific ELISA. A series of doubling dilutions of each serum sample was used in order to calculate endpoint titres using linear regression analysis. Squares represent levels of antibody in individual mice and the bar is the

median for each group. The data show that sensitization with OVA/alum leads to increased levels of anti-OVA IgE in the serum and that treatment with EtxB or with EtxB + OVA inhibits this.

Figure 7. Effects of EtxB treatment on the levels of OVA-specific IgG1 in serum

Serum levels of anti-OVA IgG1 were monitored since this IgG subclass is classically associated with a Th2 response of the type that also triggers allergy. Although IgG1 is not itself likely involved in the pathological processes underlying asthma, it is more abundant in serum and is therefore a good marker for the nature of the immune response. The data from analysing individual serum samples shows that OVA/alum sensitization triggers high levels of anti-OVA IgG1, treatment did not alter this. Squares represent levels of antibody in individual mice and the bar is the mean for each group

Figure 8. Effects of EtxB treatment on the levels of OVA-specific IgG2a in serum.

Serum levels of anti-OVA IgG2a were monitored as this subclass is classically counter-regulated with IgG1. IgG1 is promoted by IL-4 and hence Th2, while IgG2a is promoted by γIFN and hence Th1. The data indicate that OVA/alum does give rise the production of IgG2a antibodies to OVA. However, it is noteworthy that the endpoints are approximately 2 logs lower than for IgG1. This is expected since alum and the model are associated with a Th2 dominant reaction. EtxB treatment appears to reduce levels of IgG2a to a certain extent. EtxB + OVA does not affect the levels of IgG2a.

Taken together, the data clearly indicate that EtxB and EtxB + OVA are effective treatments in the OVA asthma model. All of the indicators in the lungs were favourably affected by EtxB treatment. EtxB reduced cell infiltration, particularly eosinophil infiltration. Further, EtxB reduced local IgE and associated IL-4. Analysis of other tissues revealed that the decrease in local IgE was associated with a more mild reduction in serum IgE, but not with changes to mucosal IgA and serum IgG1 levels. A slight reduction in serum IgG2a was noted. The findings also suggest that nasal EtxB

triggers the activation of regulatory T cells which then enter sites of inflammation and suppress ongoing responses there.

Fig 1: The effects of EtxB on total cell infiltration into the lung

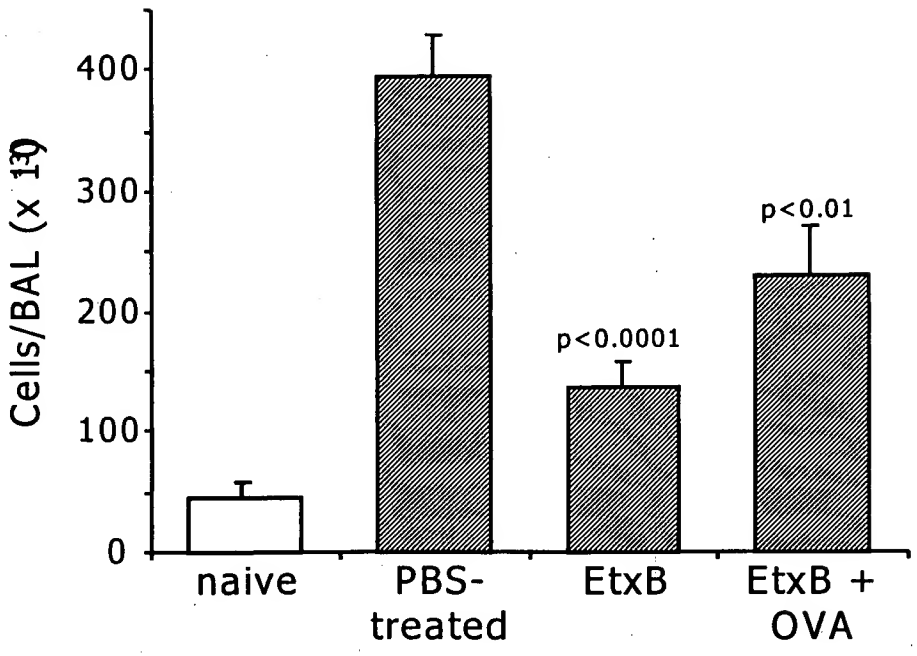


Fig 2: The effects of EtxB on cell infiltration into the lung

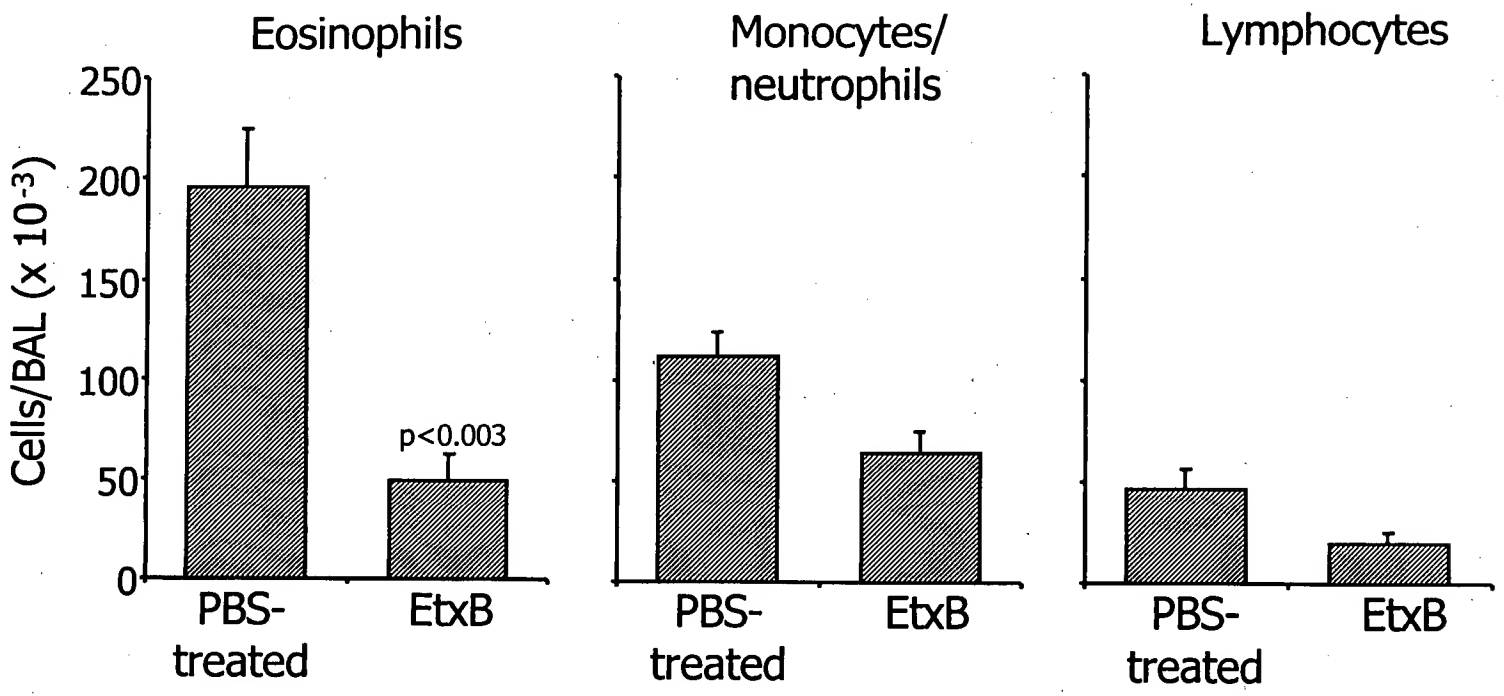


Fig 3: Effects of EtxB treatment on the levels of OVA-specific IgE in bronchoalveolar lavage fluids

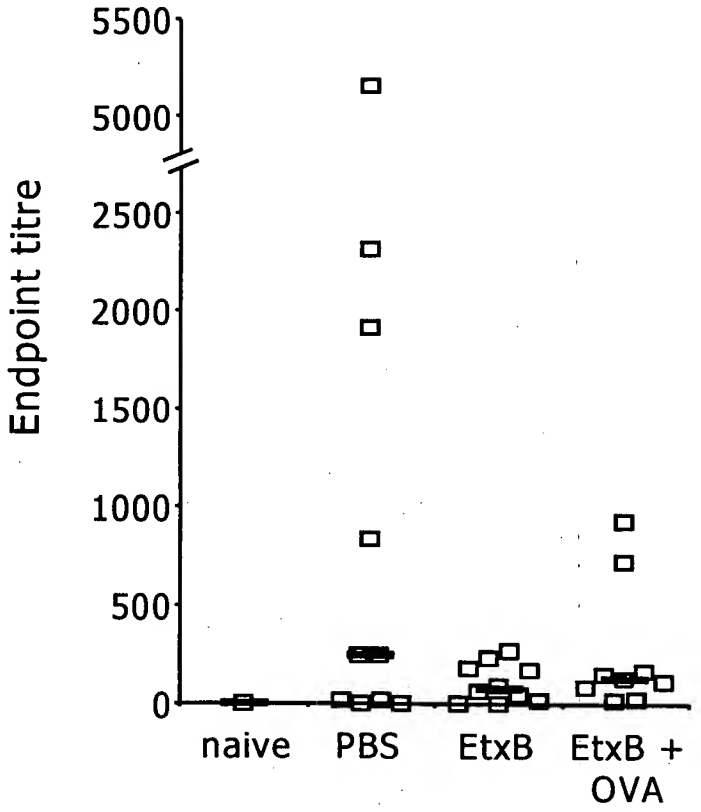


Fig 4: Effects of EtxB treatment on the levels of cytokines in bronchoalveolar lavage fluids

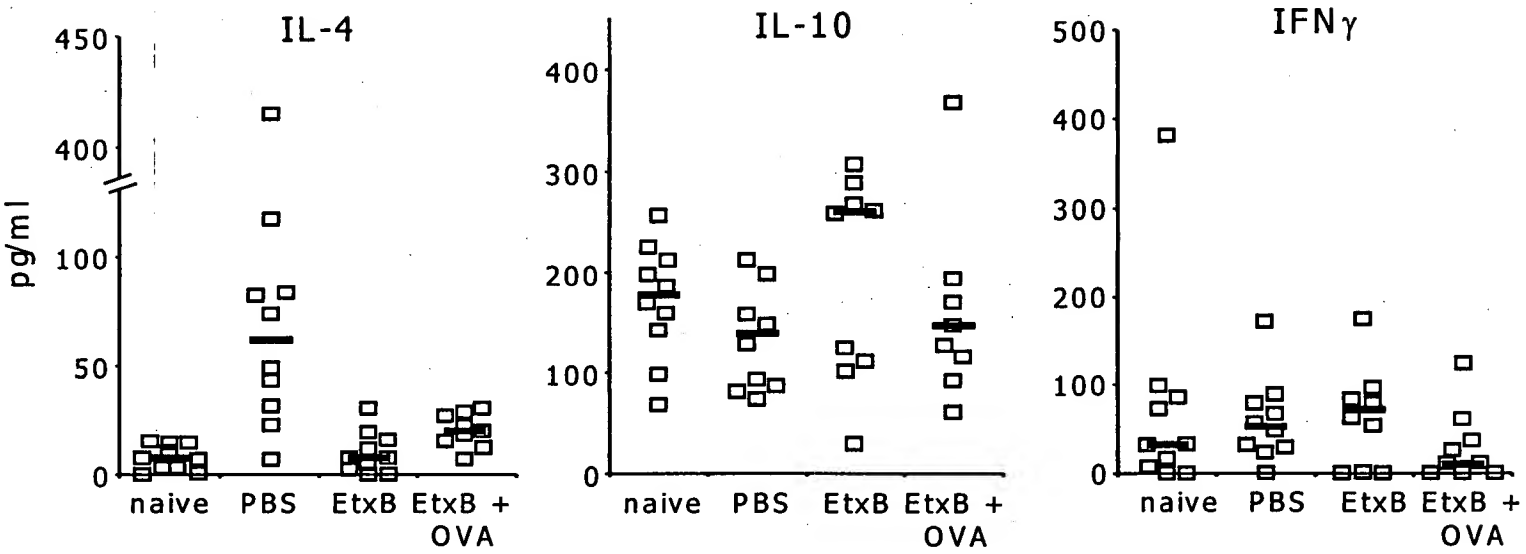


Fig 5: Effects of EtxB treatment on the levels of OVA-specific IgA in nasal washes

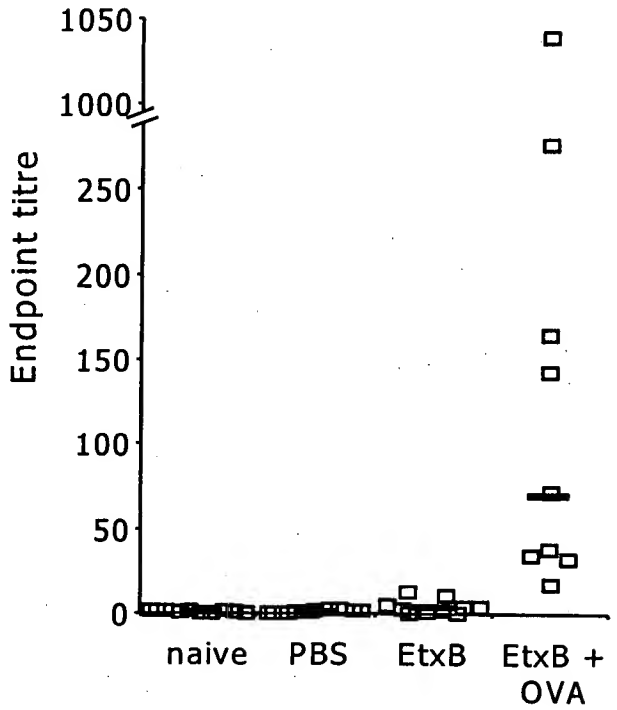


Fig 6: Effects of EtxB treatment on the levels of OVA-specific IgE in serum

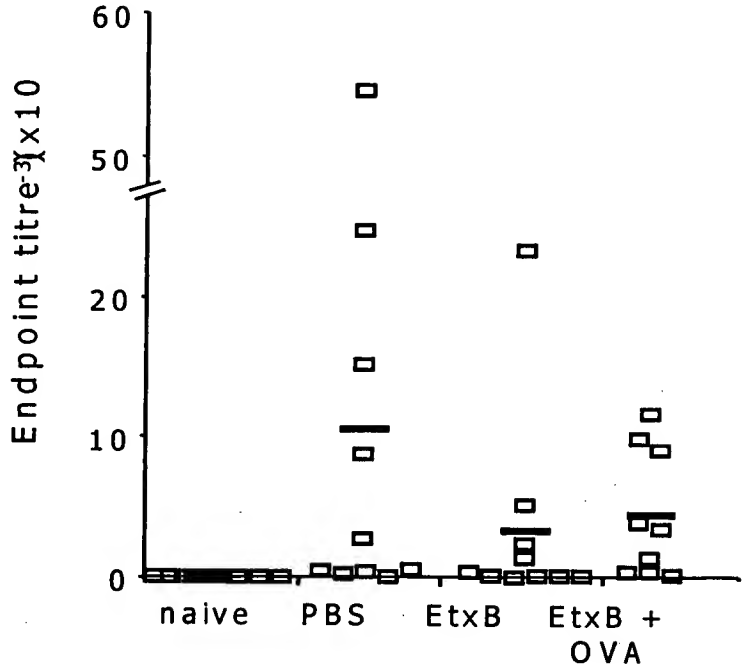


Fig 7: Effects of EtxB treatment on the levels of OVA-specific IgG1 in serum

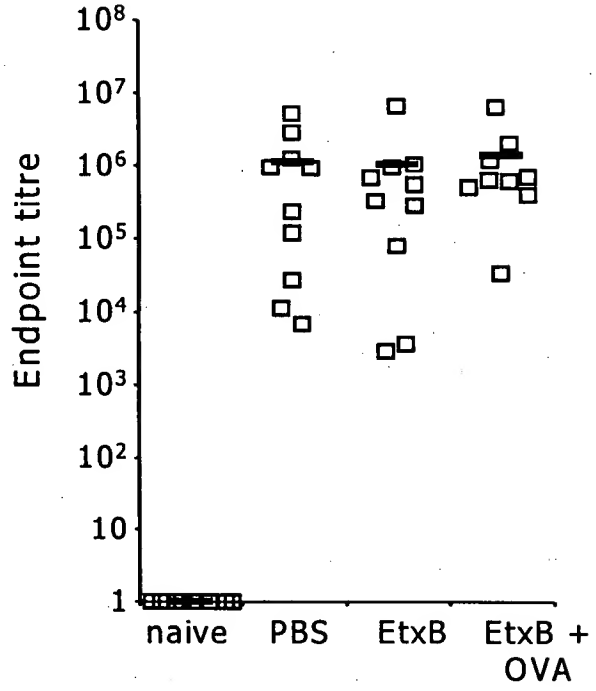
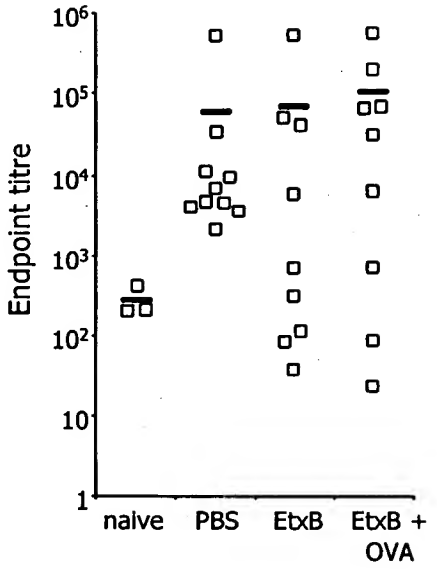


Fig 8: Effects of EtxB treatment on the levels of OVA-specific IgG2a in serum



I **Dr Neil Andrew Williams** hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issued therein.

Dr Neil Andrew Williams

16/12/02

Date 16/12/02

Dr Neil Williams is a ~~Senior Lecturer~~ Reader in Immunology at the University of Bristol, UK. He has a strong research interest in the induction and control of immune responses and has developed particular expertise in the use of the cholera-like enterotoxins as agents for vaccination and immunotherapy of autoimmune disease. The significance of his work has been widely recognised by external funding agencies with grants from the Medical Research Council, Biotechnology and Biological Sciences Research Council, The Wellcome Trust, The Commission of the European Communities, the Arthritis Research Council and the Edward Jenner Institute for Vaccine Research. Grant funds from these agencies have totalled over £2.8 million. In addition, he has worked closely with industrial sponsors, including large pharmaceutical and biotechnology companies, on collaborative programs of research receiving funding from these sources in excess of £1 million. He has published 71 scientific papers and review articles over the past 10 years. He is a regular lecturer at International conferences in Europe and the US and at academic institutions in this country and overseas.